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A STUDY ON ELECTROPHORESIS ANALYSIS OF ALPHA ESTERASE ISOZYMES DURING DIFFERENT DEVELOPMENTAL STAGES OF NEW R_1 AND R_2 RACES OF *BOMBYX MORI* L

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ABSTRACT

The molecular data, in particular gel electrophoresis of enzymes and numerical methods of analysis have proven useful in many groups of insects and will see much wider use in future. Therefore, in the current we aimed to present study was designed with the main purpose to assess the activities of alpha esterase isozymes by electrophoresis method during different developmental stages of new breeding lines R_1 and R_2races of Bombyx mori L. Standardized disc electrophoresis method was performed. Esterase isozymes form distinct enzymes zones in the photographs and in the zymogram and these have been numbered in cathodal to anodal sequence. These isozyme patterns have been established after repeated runs. The total isozymes of different developmental stages of R_1 and R_2races have been grouped into different zones. The nomenclature of enzyme banding pattern has been followed. Study findingsdelineated9 bands in race R_1 and 6 bands in race R_2 were recorded for alpha esterase and all the bands were strongly resulted in R_1 and R_2 during embryogenesis. At larval stages 14 and 18 bands are recorded for alpha-esterases in R_1 and R_2 races. There is a gradual increase of esterase isozymes from eggs to pupae and they decreased in adults. The developmental esterases show a gradual increase in the number of isozymes from I larval to V larval instar of all the races studied viz. R_1 and R_2 races. Furthermore, results of the esterase activity in pupal stage of the two races studied indicate that alpha non-specific esterases are absent in R_1 and R_2. However, high specific esterase activity was noticed in R_1 and R_2 races.

KEYWORDS: Bombyx Mori L, Electrophoresis, Alpha Esterase, Races, Pupa, Larva

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Original Article

INTRODUCTION

A concept of study on the taxonomy of closely related species, studies on the entomological taxonomy based on morphologically defined species, the degree of morphological difference, and topological approacheshave failed to deal adequately with sibling or cryptic species (Davidson et al., 1967). In such cases the use of biochemical study has been considered of some use in the separation of closely related species of insects.

Literature reports evidenced that reliable methods for distinguishing members of the insect complex by chromatographic studies of pteridine species have been attempted which help to identify the members of the complex (Davidson et al., 1967, Micks et al., 1966 a, b Micks 1968). Immuno diffusing techniques have been used to separate insect species in particular mosquitoes (Cupp and Ibrahim, 1973, Cupp et al., 1970). Schumann (1973) analysed through geldiffusion techniques different strains of mosquitoes of different origin and identified them.

A science concerned with establishing durable classification has itself undergone tremendous changes in the last three decades. Traditional approach still is the basis of all taxonomical studies. The molecular data, in

particular gel electrophoresis of enzymes (Yashitake 1963 1968, Eguchi 1965, 1968 Kai and Nishi 1976 Takeda et al., 1992), and numerical methods of analysis have proven useful in many groups of insects and will see much wider use in future (Berlacher 1984). With this scenario, in the current we aimed to analyze the activities of alpha esterase isozymes by electrophoresis method during different developmental stages of new races R_1 and R_2 of *Bombyx mori* L.

MATERIALS AND METHODS

Silkworm Varieties and Rearing

The pure races of bivoltine Kalimpong-A (KA) spinning oval white cocoons, New Bivoltine-18 (NB_{18}) spinning dumbbell white cocoons and multivoltine Pure Mysore (PM) spinning pointed yellow cocoons of mulberry silkworm Bombyx *mori* L. were selected for the present breeding programme. These races were obtained from their respective seed areas and are reared in cytogenetics laboratory, Jnana Bharathi, Bangalore University.

The disease free layings were prepared as described by Krishnaswamy, and were incubated at 25°C and relative humidity of 60-70%. On 8th day composite layings were prepared (10-20 layings were prepared 100-200 eggs were collected from each laying). The hatched worms were reared according to the method described by Krishnaswamy (Krishmaswamy, 1978). MS variety of mulberry leaves were used in rearing. The worms were reared in mass up to III instar, after III moult 300 worms were collected in three replicates in order to evaluate the rearing performance. Standard temperature and humidity were maintained in the rearing house.

Breeding

Single and three way crosses were made by using the above said three races. The first single cross involved KA females and PM males. The second single cross involved NB_{18} females and PM males. During the course of breeding selection was made at the egg, larva, pupa and cocoon stages to fix the desirable traits. F_5 progenies of the respective crosses were back crossed to their respective bivoltine males to improve commercial characters.

Evolutions of New Lines R_1 and R_2

Females of KA and NB_{18} were crossed with males of PM. The composite layings of F_1 hybrid were brushed and reared under standard laboratory conditions. The selection parameters explained earlier were applied to choose the seed cocoons for the preparation of F_2 layings. The replicates showing higher pupation rate were selected for intra family selection of cocoons. Further, segregation with respect to cocoon colour and built was noticed. Only white oval in case of KAxPM and dumbbell white in case of NB_{18}xPM qualifying the parameter of selection were choosen for breeding in subsequent generations. The females of F_5 were backcrossed to the males of KA and NB_{18} respectively in both the lines and reared up to 11 generations. At the end of the 11th generation the lines R_1 and R_2 were extracted with higher ERR than their respective better parents, with shorter larval period and with moderate cocoon productivity character in case of R_1 and R_2.

Table 1

Breeding Plans I and II													
				I							**II**		
	KA	O	O	x	PM	Cto		NB18	O	O	x	PM	Cfo
		+	i-						+	+			
				F1							F1		
				F2							F2		
				F3							F3		
				F4							F4		
F5	x	KA	O	➘	er'		F5x	NB18	Cta	+			
				F1							F1		
				F2							F2		
				F3							F3		
				F4							F4		
				F5							F5		
				F6	(R1)						F6	(R2)	

Preparation of Enzyme Extract

The different developmental stages such as 1st day, 5^{th} day and 9th day eggs, five larval instars (I, II, III, IV, and V instars), early, middle and late stages of male and female pupae, male moths before and after copulation. Female moths before and after egg laying of bivoltine races KA, NB_{18}, and multivoltine race PM were selected.

Electrophoresis

Disc electrophoresis was performed essentially according to Davis (1964) and Ornstein (1964). A discontinuous gel system consisting of 7.5% lower gel and 3.12 5% spacer gel was used. The lower gel consisted of one part of Tris-hydrochloric acid buffer (36.g Tris+ 48.0 ml of N HCl + 0.46 ml of TEMED, diluted to 100ml. pH 8.9), two parts of cyanogum 41 (3.08 g of cyanogum in 10ml of water), two parts of Ammonium persulphate (140mg of APS in 100ml of water) and three parts of distilled water. 1.2 ml of this solution was poured into clean, dry glass tubes (7 cm x 0.7 cm dia) held vertically. The solution was carefully over layered with distilled water and allowed to photopolymerise for 15 minutes under fluorescent lamp or day light. After polymerisation, the water layer was removed from the top and the spacer gel was added. The spacer gel consisted of 1 part of Tris phophoretic acid buffer (5.7 g tris + 25.6 ml of 1M H3Po4 + 0.46 ml of TEMED diluted to 100 ml with distilled water pH 6.9) 2 parts of cyanogen 41 (1.25 g cyanogum 41 in 10ml of Distilled water) 1 part of APS (70 mg in 100 ml) and four parts of water. 0.2 ml of spacer gel was poured on the top of the lower gel each tube layered with a drop of water and allowed to Photopolymerise for 15 minutes. After polymerization the water was blotted off and the tubes with spacer gel were inserted into the rubber connectors of the upper electrode vessel. The electrode chambers were filled with electrode buffer (0.3 M boric acid and sodium hydroxide buffer pH 8.65). The sample, suitably diluted with 20% sucrose containing bromophenol blue, was carefully layered on to each gel and subjected to electrophoresis in cold (4°C) imposing a current of 2mA per tube for 2 hours.

Staining Procedure

The staining techniques of Ayala et al. (1972) was followed with a slight modification. The stain used for esterases constituted 25 mg of alpha naphthyl acetate dissolved in 2 of 1:1 acetone water and the same was added to 12.5 ml of 0.1 M phosphate buffer pH 5.9 to which 25 mg of Fast blue RR salt and 12.5 ml of 0.1 M phosphate buffer pH 6.5 were added. For beta-esterase the same incubating medium was used except for the substrate where beta-naphthyl acetate was substituted in place of alhpa salt. The gels were incubated in the stain for 30 minutes until the bands appeared. The gels

were then stored in 6% acetic acid. Esterase isozymes form distinct enzymes zones in the photographs and in the zymogram and these have been numbered in cathodal to anodal sequence. These isozyme patterns have been established after repeated runs. The total isozymes of different developmental stages of KA, NB_{18}, PM, R_1 and R_2 have been grouped into different zones. The nomenclature of enzyme banding pattern has been followed after Ayala et al. (1972). The relative front (Rf) of the esterase and phosphatase bands of all the developmental stages with reference to known indicator dye was calculated as follows:

Rf = (Length gel before staining/ Length gel after staining)

X

(Distance moved by band/Distance moved by marker dye)

RESULTS

Electrophoretic analysis of alpha-esterase isozymes in different developmental stages of silkworm Bombyx mori races NB_{18}, KA, PM and newly evolved races R_1 and R_2 have revealed marked variation in the number of isozymes. The photographs and zymograms reveal the presence of 21 isozymes based on their relative front values. The alpha-esterase isozymes found in different developmental stages of the above species are classified into eight esterase zones. They are Est-1, Rf 0.06-0.12, Est-2 Rf 0.2-0.23; Est-3 Rf 0.30; Est-4 0.41-0.51; Est-5 Rf 5.5-6.2; Est-6 Rf 0.65-0.71; Est-7 Rf 0.76-0.83; Est-8 Rf 0.91-1.0. The isozyme patterns are numbered in a cathodal to anodal sequence from 1-21.

The alpha-esterase zymograms of the isolated line R_1 are grouped into 8 esterase zones. Est-1 zone consists of two bands (1 and 2). Band 1 1s absent and band 2 is darkly stained in I instar larvae and is specific. Est-2 zone consists of 2 bands (3 and 4). Band 3 1s moderately stained in III instar larvae and 24h male pupae, 1s darkly stained in II and larvae. Band 4 is darkly stained 1n II and V instar larvae. Est-3 zone consists of 3 bands (5, 6 and 7). Band 5 1s moderately stained in 24h male pupae and 144h female pupae, darkly stained 1n 24h eggs, I, V instar larvae, 288h female pupae. Band 6 1s darkly stained in I and V instar larvae. Band 7 is moderately stained in 288h male pupae, darkly stained in I, III, V instar larvae and 24h male pupae. Est-4 zone consists of 3 bands (8,9 and 10). Band 8 1s moderately stained in 144h and 288h male pupae, darkly stained in 120h, 216h eggs, III, IV instar larvae. Band 9 is moderately stained 1n 144h male pupae and 24h female pupae, darkly stained in 120h, 216h eggs, III instar larvae. Band 10 1s moderately stained in 288h female pupae, darkly stained 216h eggs, I instar larvae, V instar larvae, 24h male pupae and female adults before oviposition. Est-5 zone consists of 3 bands (11,12 and 13). Band 11 1s moderately stained 1n 24h female pupae, darkly stained 1n I, IV instar larvae and female adult before oviposition.Band 12 is moderately stained in 144h 288h female pupae, male adult before copulation, darkly stained in 24h eggs, II, III, IV instar larvae, 288h male pupae and female adult before oviposition. Band 13 is darkly stained in 120h eggs, II, III and V instar larvae, 24h, 288h male pupae.

Est-6 zone consists of 2 bands (14 and 15). Band 14 is moderately stained in 144h, 288h female pupae and male adult after copulation, darkly stained in 120h, 216h eggs IV and instar larvae. Band 15 is moderately stained in 288h male pupae, 24h female pupae and female adult before and after oviposition, darkly stained in 120h eggs, IV instar larvae, 24h male pupae 144h female pupae. Band 15 is moderately stained in 288h male pupae, 24h female pupae and female adult before and after oviposition, darkly stained in 120h eggs, IV instar larvae, 24h male pupae, 144h female pupae. Est-7 zone consists of 3 bands (16, 17 and 18). Band 16 is faintly stained in II instar larvae, moderately stained in 24h female pupae and female adult before and after oviposition, darkly stained in V instar larvae, 144h, 288h female pupae, male adult before

copulation. Band 17 is darkly stained in V instar larvae, 24h, 144h, 288h male pupae male adult before and after copulation and female adult after oviposition. Band 18 is darkly stained in 24h, 288h male pupae, 288h female pupae, male adult before and after copulation female adult before and after oviposition.

Est-8 zone consists of 3 bands (19,20 and 21). Band 19 is darkly stained in V instar larvae, male adult before and after copulation, female adult before oviposition. Band 20 is likely stained in 24h male pupae, female adult before and after oviposition. Band 21 is present in all the developmental stages (Figure 1, 2, and 3).

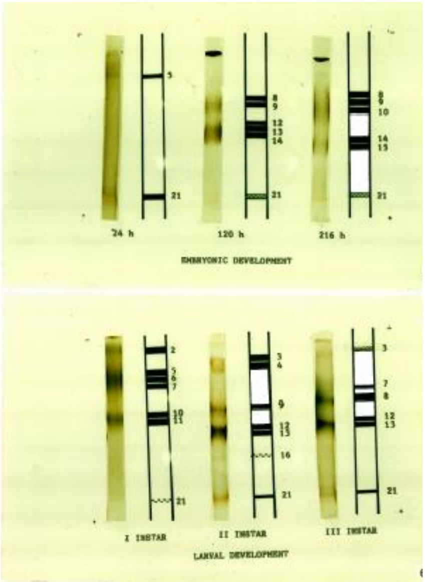

Figure 1: Alpha Esterase Zymograms of R_1.

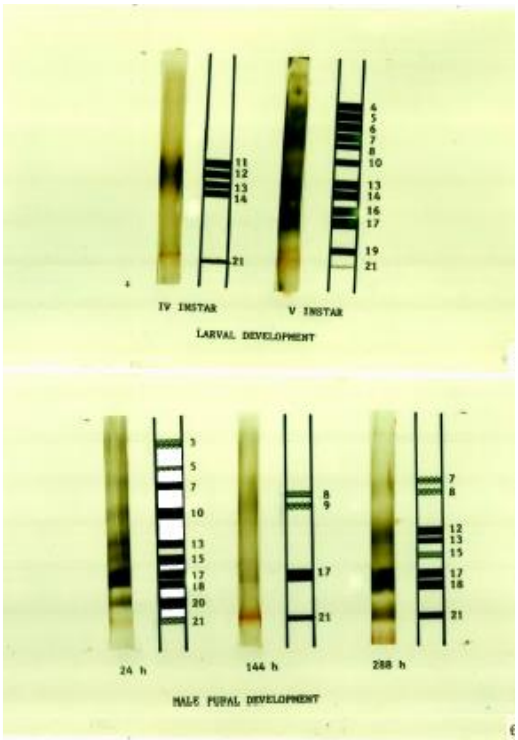

Figure 2: Alpha Esterase Zymograms of R_1.

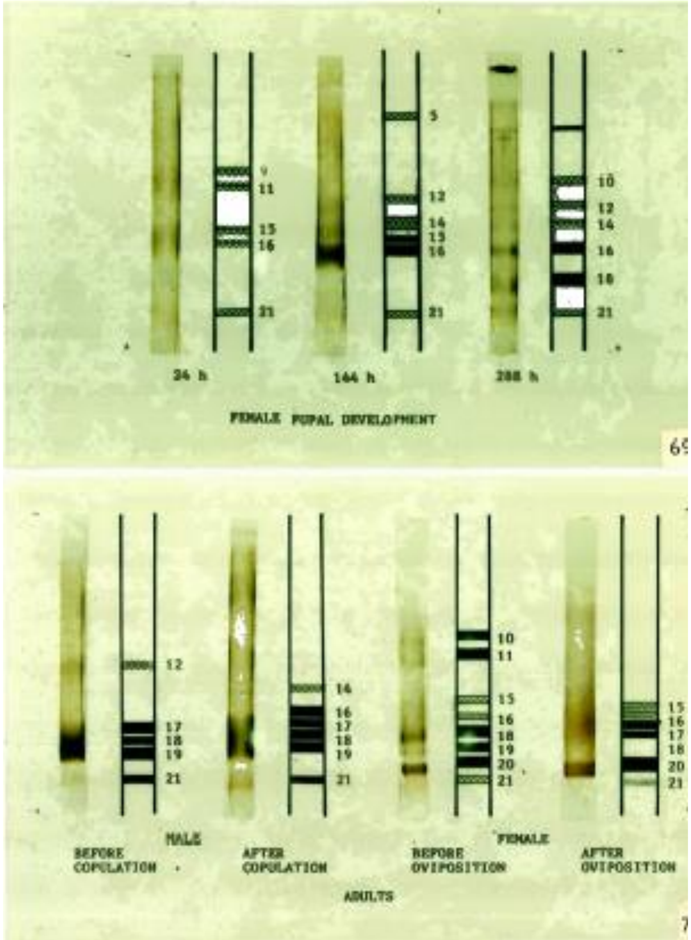

Figure 3: Alpha Esterase Zymograms of R_1.

The alpha-esterase zymograms of the isolated line R_2 are grouped into 8 esterase zones. Est-1 zone consists of 2 bands (1 and 2). Band 1 is absent in all the stages. Band 2 is partly stained in II instar larvae and 24h female pupae and female adult after oviposition. Est-2 zone consists of 2 bands (band 3 and 4). Band 3 is darkly stained in II instar larvae, 24h female pupae, male adult after copulation. Band 4 is moderately stained in III instar larvae, darkly stained in II instar larvae, female pupae 24h and 288h. Est-3 zone consists of 3 bands (5,6 and 7). Band 5 is faintly stained in 144h female pupae, moderately stained in I, instar larvae, 24h, 144h male pupae, darkly stained in 288h female pupae and male adult after copulation. Band 6 is moderately stained in I and V instar larvae, darkly stained 1n III instar larvae, 24h female pupae and female adult after copulation. Band 7 is moderately stained in male adult before copulation, darkly stained in 288h male pupae, 24h fen ale pupae, male adult after copulation and female adult after oviposition.

Est-5 zone consists of 3 bands (8,9 and10). Band 8 1s moderately stained 1n 144h female pupae and 1s darkly stained 1n I, II instar larvae, 24h, 144h, 288h male pupae and 288h female pupae, male adult after copulation and female adult before oviposition. Band 9 1s faintly stained in IV instar larvae, darkly stained in I instar larvae, 24h, 144h male pupae, 24h female pupae, 288h female pupae and female adult before oviposition. Band 10is darkly stained in V instar larvae, 288h male pupae, 24h female pupae and female adult after oviposition. Est-6 zone consists of 2 bands (14 and 15). Band 14 1s present 1n 288h male pupae. Band 15 1s faintly stained in IV instar larvae, moderately stained 1n III instar larvae, darkly stained in 288h female pupae and male adult before copulation. Est-7 zone consists of 3 bands (16,17 and 18). Band 16 1s moderately stained 1n V instar larvae, darkly stained 1n IV instar larvae and male adult before copulation.

Band 17 1s darkly stained in 24h eggs and 288h female pupae. Band 18 is faintly stained in adult before and after oviposition, moderately stained in male adult after copulation and is darkly stained in 24h, 120h and 216h egg, 288h male pupae, 288h female pupae, male pupae, 288h female pupae, male adult before copulation. Est-8 zone consists of 3 bands (19,20 and 21). Band 19 is faintly stained in male adult after copulation, darkly stained in V instar larvae, 24h, 120h and 216h egg, male pupae 288h. Band 20 is darkly stained in 24h, 216h egg. Band 21 is present in all developmental stages (Figure 4, 5, and 6).

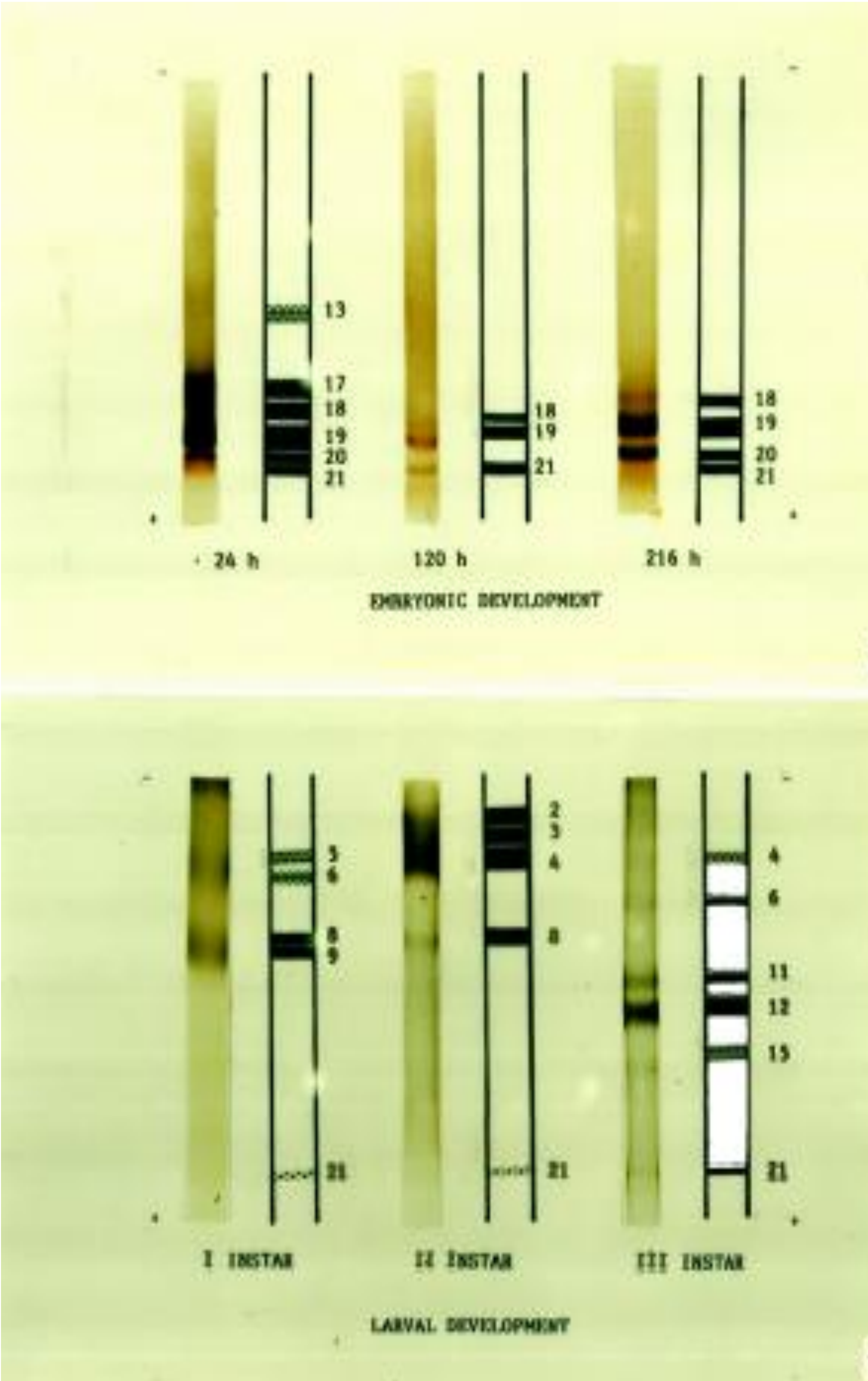

Figure 4: Alpha Esterase Zymograms of R2.

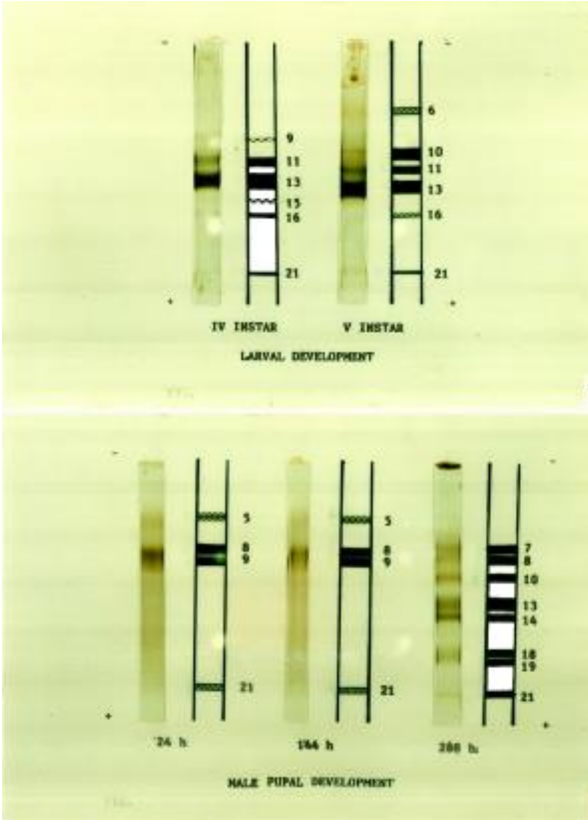

Figure 5: Alpha Esterase Zymograms of R2.

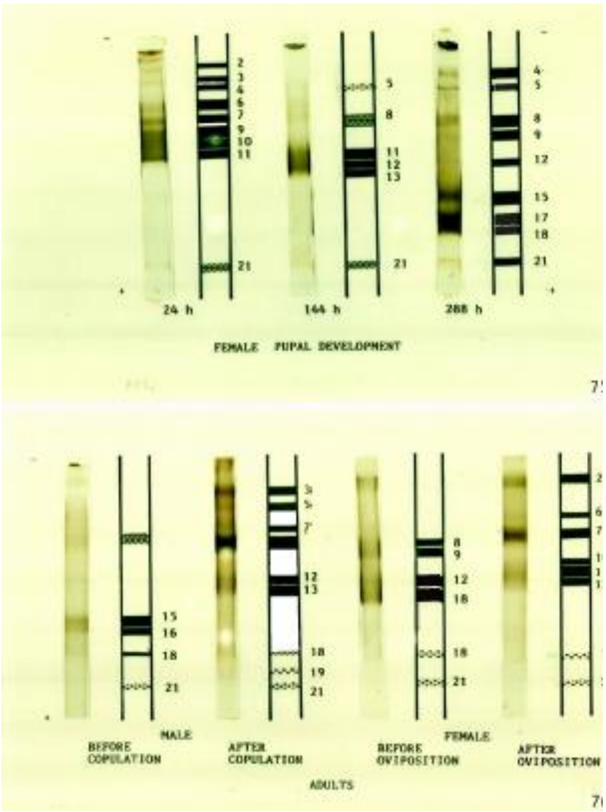

Figure 6: Alpha Esterase Zymograms of R2.

DISCUSSION

In the current study 9 bands in R_1 and 6 bands in R_2 were recorded for alpha esterase and all the bands were strongly resulted in R_1 and R_2 during embryogenesis. These findings were in accordance with the report of Fei and Sheng (1987) wherein authors reported a total of 12 esterase isozymes during embryogenesis. More or less same investigations have been conducted on the eri silkworm *Philosamia ricini* (Revanasiddaiah et al., 1989), and they reported 30 bands at various developmental stages. But their studies were conducted around egg development and they noticed twelve isozymes in embryogenesis.

At larval stages 14 and 18 bands are recorded for alpha-esterases in R_1 and R_2 races. In pupal stages more number of bands are recorded and in adults the numbers have been decreased. There is a gradual increase of esterase isozymes from eggs to pupae and they decreased in adults. This variation reflects regulation of gene activity so as to meet the den and of different metabolic activities (Revanasiddaiah et al., 1989, Krishnamurthy al., 1984).

The eggs show high esterase activity. This is due to the presence of large amounts of enzymes stored in the yolk which will be utilized during embryogenesis. The larval, pupal and adult stages reveal maximum heterogeneity like *P. ricini*. Esterase isozymes show sexual dimorphism in both pupal and adult stages. This shows stable difference in the expression of different genes in the same race of *B. mori* during ontogeny.

Thus the analysis of isozymes at different developmental stages of pure races and their isolated races suggest that the enzyme bands appear, disappear and reappear in different developmental stages. On the basis of their manifestation, the bands have been classified into two categories. One category persists throughout the developmental stages and called "generalized segregating and non-specific esterases" which include Est-3, Est-4, Est-5, Est-6 and Est-7 zones of alpha esterase. These are comparable to the results found in Drosophila 1mmigrans (Pautelouris and Downer, 1969).

The other category which is confined to some of the developmental stages is called "specific non segregating esterases" which include Est-1, Est-2 and Est-8 of alpha esterase. These are comparable to the ones found in *Zaprionus paravittiger* described by Kaur and Parkash (1979). The electrophoretic analysis has shown that the eggs of all the R_1 races contain alpha Est-3, Est-4, Est-5 zones. Whereas in R_2 races only the specific esterase zone persists and all other esterases are completely absent. The developmental esterases show a gradual increase in the number of isozymes from I larval to V larval instar of all the races studied *viz.* R_1 and R_2 races. Such an increase during development has been reported in Drosophila nastuta by Siddaveeregowda et al. (1977) and also insects in general by Laufer (1961). A comparative study pertaining to larval developmental stages of the races show a gradual increase in the number of isozymes from I larval to V larval instar in R_1 and R_2races.

The results of the esterase activity in pupal stage of the two races studied indicate that alpha non-specific esterases are absent in R_1 and R_2. However, high specific esterase activity was noticed in R_1 and R_2 races. This observation coincides with that of Prakash and Reddy (1978) who have reported such high activity of alpha esterase in the pupal stage of fruit fly Drosophila rajashekari.

CONCLUSIONS

In conclusion, results of the current study delineated 9 bands in race R_1 and 6 bands in race R_2 were recorded for alpha esterase and all the bands were strongly resulted in R_1 and R_2 during embryogenesis. At larval stages 14 and 18 bands are recorded for alpha-esterases in R_1 and R_2 races. There is a gradual increase of esterase isozymes from eggs to pupae and

they decreased in adults. The developmental esterases show a gradual increase in the number of isozymes from I larval to V larval instar of all the races studied *viz.* R_1 and R_2 races. Furthermore, results of the esterase activity in pupal stage of the two races studied indicate that alpha non-specific esterases are absent in R_1 and R_2. However, high specific esterase activity was noticed in R_1 and R_2 races.

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